

## Standard Paper

# Morphological and phylogenetic analyses of *Toniniopsis subincompta* s. lat. (Ramalinaceae, Lecanorales) in Eurasia

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## Abstract

In recent years, several species that have long been considered to belong in *Bacidia* s. lat. have been transferred to other genera such as *Bellicidia*, *Bibbya*, *Scutula*, and also to *Toniniopsis*, accommodating species previously placed in *Bacidia* and *Toninia*. One of its widespread species, *Toniniopsis subincompta*, can be recognized by its thinly granular thallus, dark brown to black apothecia, green epithecium, red-brown hypothecium, and bacilliform ascospores. However, it shows considerable variation in thallus structure, and coloration of apothecia, hypothecium and exciple. We sequenced 20 specimens of *T. subincompta* to investigate whether there is phylogenetic support for the delimitation of species in accordance with the variability of the observed characters. For phylogenetic analyses, we used newly generated sequence data from the nuclear internal transcribed spacer (nrITS), mitochondrial small subunit (mtSSU) and DNA-directed RNA polymerase II subunit (*RPB2*). Maximum likelihood and Bayesian inference analyses, as well as three species delimitation programs, provided consistent evidence that *T. subincompta* forms two separate lineages, to be recognized at the species level. The complex nomenclature of *T. subincompta* (basionym *Lecidea subincompta*) shows it to be a synonym of *Bellicidia incompta*. For the most common taxon previously called *Bacidia (Toniniopsis) subincompta*, the new combination *T. separabilis* is made, rather than proposing a conserved type for *Lecidea subincompta*. *Toniniopsis dissimilis* is newly described to accommodate the less common taxon. *Toniniopsis dissimilis* is characterized by a predominantly wrinkled to warted to subsquamulose thallus; generally grey-brown to dark brown apothecia, often with a lighter margin; a dark brown hypothecium, frequently gradually merging into the coloration of the exciple below and the lateral part of the exciple attached to the hymenium; a mostly colourless rim and lateral part of the exciple. The closely related *T. separabilis* is characterized by a thallus of mostly single or contiguous ± loose granules, often forming short, coralloid, isidium-like bulges; darker apothecia, with a margin mostly of the same colour or darker than the disc; a comparatively thinner hypothecium easily separated from the exciple below. The rim and lateral part of the exciple often contain either a blue, brown or mixed blue-brown colour in the upper part or along the whole margin. Lectotypes of *Bacidia vegeta*, *Lecidea bacillifera* f. *melanotica* and *Secoliga atrosanguinea* var. *affinis* (the synonyms of *T. separabilis*) are selected. Cyanotrophy and the occurrence of albino morphs in *T. separabilis* are discussed.

**Keywords:** albino morph, *Bacidia* s. lat, cyanotrophy, lichen, phylogeny, species delimitation, taxonomy

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## Introduction

Until recently, a large variety of lichens were accommodated in the genus *Bacidia* in the broad sense. Zahlbruckner (1905) used the genus name for crustose lichens with a chlorococcoid photobiont, biatorine apothecia, and ascospores with three or more transverse septa. This circumscription was apparently unnatural and included numerous taxa that are not closely related to the type species, *Bacidia rosella* (Pers.) De Not. (Gerasimova & Ekman 2017). In recent phylogenetic studies, several species that have been considered within *Bacidia* for a long time have

been transferred to other genera such as *Bellicidia* Kistenich *et al.*, *Bibbya* J. H. Willis, *Scutula* Tul. and *Toniniopsis* Frey (Kistenich *et al.* 2018). Moreover, phylogenetic results showed that *Toninia* A. Massal. was paraphyletic and *Bacidia subincompta* (Nyl.) Arnold, *Toninia aromatica* (Sm.) A. Massal. and *T. coelestina* (Anzi) Vězda grouped with *Toniniopsis illudens* (Nyl.) Kistenich *et al.* (formerly *Bacidia illudens* (Nyl.) H. Olivier). *Toniniopsis* is morphologically similar to *Toninia* but differs in the stronger pigmentation of the exciple and the presence of a blue-green pigment in the hymenium and sometimes also in the proper exciple.

A widespread species of the genus, *T. subincompta* (Nyl.) Kistenich *et al.*, is a corticolous species, occurring in Europe, Asia, Macaronesia, Africa and North America. It occurs in a range of woodlands from sea level to an altitude of c. 3000 m (Ekman 1996; Coppins & Aptroot 2009). It is also used as an

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indicator for old-growth forest and is included in indices for determining the conservation importance of woodland areas (Rücker & Wittmann 1995; Rose & Coppins 2002; Coppins & Aptroot 2009; Brackel 2019). *Toniniopsis subincompta* can generally be recognized by its thinly granular thallus, black apothecia, green epithecium, red-brown hypothecium, and bacilliform ascospores (Coppins & Aptroot 2009). However, it can vary in spore shape, including both bacilliform and acicular spores, and in thallus structure, ranging from either discontinuous (discrete granules or low convex areoles) to continuous (without cracks or ±rimose, wrinkled, warty, tuberculate or distinctly granular) (Ekman (1996) as *Bacidia subincompta*). Further detailed examination has revealed differences in coloration of the apothecia (including a pale albino morph (Gilbert 1996)), hypothecium and exciple, in addition to the thallus and ascospore variation.

The aim of this paper is to clarify and re-evaluate the taxonomy of the morphologically variable *T. subincompta* s. lat. using different approaches based on material collected from the mountain regions of Allgäu (Germany) and the North Caucasus (Russia), where *T. subincompta* is well represented at different elevations and on various substrata. Here we present a three-locus phylogeny of *T. subincompta* s. lat. and assess the species morphology based on an enlarged taxon sampling and detailed observation of type specimens. Furthermore, we applied several species delimitation programs in order to test for congruence of species differentiation based on different analyses of the molecular data.

## Material and Methods

We included specimens from Germany (Allgäu mountain region; eight specimens), Russia (the North Caucasus as well as the Altay, Kaliningrad and Murmansk regions; ten specimens) and Estonia (Valgamaa and Harjumaa; two specimens) collected at different elevations and bark substrata, in the period 2013–2018 (Table 1). To test if dark and albino morphs belong to the same species, we selected specimens of both morph types from the bark of the same tree (JG145 and JG146). Voucher specimens are deposited in the herbaria of the Botanische Staatssammlung München (M), University of Tartu (TU) and Komarov Botanical Institute (LE). The herbarium collection of *T. subincompta* s. lat. (55 specimens) stored in the Botanical Collection of Munich (M) was also studied.

## Morphology

Microscopic observations were made using a Zeiss Axioplan light microscope (Oberkochen, Germany) equipped with differential interference contrast (DIC). Cross-sections of apothecia were made on a Leica Jung Histoslide 2000 Mikrotom (Heidelberg, Germany), with a thickness of 8–10 µm. Micrographs of cross-sections were taken on a Zeiss Axioplan with an attached AxioCam 512 Color camera and processed with the Zeiss ZEN 2.3. (blue edition) image program. Macrographs of external characters were taken on a Leica Z6 Apo microscope (with a ×2.0 Planapo lens; Leica, Germany) with a Sony Alpha 6400 camera (Sony, Japan) attached and equipped with a StackShot Macro Rail (Cognisys, USA). A single image was prepared from 30–40 serial images using Helicon Focus v.7 software (Helicon, USA).

Measurements are given as (min–) average ± SD (–max) (SD = standard deviation,  $n_1$  = number of all observations,  $n_2$  = number of specimens observed). We provide a detailed description of specimens using standard microscopic techniques following Coppins & Aptroot (2009) and the subdivision scheme of the proper

exciple according to Ekman (1996), differentiating the following structures: the rim, lateral part and medullary part. To delimit the species lineages, we discuss the following diagnostic characters in detail: 1) thallus structure; 2) colour of disc and margin of apothecium; 3) colour of hypothecium; 4) colour and structure of exciple; 5) shape and size of ascospores. Pigment characterizations follow Meyer & Printzen (2000).

## DNA extraction, PCR amplification and DNA sequencing

DNA extraction was carried out using the Stratec Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) following the manufacturer's instructions. Five to eight apothecia were used from fresh material not older than 5 years and thallus fragments were removed in order to minimize the risk of contamination by, for example, lichenicolous fungi. PCR amplification, purification and sequencing were performed as described in Gerasimova *et al.* (2018). Cycling conditions included initial denaturation at 95 °C for 2 min, 5 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, 33 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, and a final extension step at 72 °C for 7 min. In those cases when the PCR product was not sufficient, a second PCR with a reduced number of cycles was conducted: denaturation at 95 °C for 2 min, 5 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, 22 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, with a final extension step at 72 °C for 7 min. We used the primers ITS1F (White *et al.* 1990) and ITS4m (Beck & Mayr 2012), mtSSU1 and mtSSU3R (Zoller *et al.* 1999), and rRPB2-5F and rRPB2-7cR (Liu *et al.* 1999).

## Alignment and phylogenetic analyses

In addition to 57 (18 nrITS, 19 mtSSU and 20 RPB2) newly obtained sequences of *Toniniopsis subincompta* s. lat., we included sequences of *Toniniopsis* and *Toninia* from GenBank in our alignment when at least two loci were available (published in Ekman 2001; Kistenich *et al.* 2018). In an additional analysis, we added to our alignment six further nrITS sequences of *Toniniopsis subincompta* from GenBank, collected in Switzerland (Mark *et al.* 2016) and Norway (Ekman 2001; Kistenich *et al.* 2018), in order to examine their distribution among specimens from other localities in our phylogeny (results are discussed below, and see Supplementary Material File S1, Figs S2–S4, available online).

BLAST searches in GenBank were performed to detect and exclude accessory/lichenicolous fungi and potential contaminations. Alignments were carried out using standard settings in MUSCLE v.3.8.31 (Edgar 2004) as implemented in PhyDE-1 v.0.9971 and optimized manually. Positions, where a gap had to be inserted in more than 95% of the sequences, were excluded. The alignment with nrITS, mtSSU and RPB2 was subjected to Bayesian inference (BI) and maximum likelihood (RAxML and IQ-TREE) analyses for the single and concatenated datasets separately.

Bayesian inference was carried out using the Markov chain Monte Carlo method (MCMC) using MrBayes v.3.2.6 (Ronquist *et al.* 2012). The GTR substitution model with gamma-distributed rate variations across sites and a proportion of invariable sites was selected by jModelTest 2.1.10 v20160303 (Darriba *et al.* 2012) based on the Akaike Information Criterion. Two parallel runs were performed (two cold chains) with a single tree saved every 10th generation for a total of 1 000 000 generations. The initial 25% was discarded as burn-in and the results are summarized as a 50% majority-rule consensus tree.

**Table 1.** Specimen information and DNA codes for *Toninia* and *Toniniopsis* samples used in this study, with their respective GenBank Accession numbers. New sequences are in bold.

DNA no. (JG)	Name	Country	Locality	Specimen voucher	GenBank Accession number		
					nrITS	RPB2	mtSSU
	<i>Toninia cinereovirens</i>	Norway	no indication	Haugan & Timdal 7953 (O)	AF282104	AM292781	AY567724
	<i>T. squalida</i>	Norway	no indication	Haugan 4970 (O)	AF282103	MG926297	MG925940
	<i>Toniniopsis aromatica</i>	Norway	no indication	Haugan & Timdal 4819 (O)	AF282126	MG926285	MG925926
	<i>T. coelestina</i>	Norway	no indication	Haugan 5985 (O)	AF282127	MG926291	MG925933
	<i>T. dissimilis</i>	Norway	Finnmark, Batsfjord	O-L-170623	MG838157	–	–
<b>148</b>	<i>T. dissimilis</i>	Germany	Oberallgäu, Markt Oberstdorf	Gerasimova & Beck M-0290431 (M)	<b>MT169977</b>	<b>MT180447</b>	<b>MT162221</b>
<b>149</b>	<i>T. dissimilis</i>	Germany	Oberallgäu, Markt Oberstdorf	Gerasimova & Beck M-0290432 (M)	<b>MT169978</b>	<b>MT180448</b>	<b>MT162222</b>
<b>150</b>	<i>T. dissimilis</i>	Germany	Oberallgäu, Markt Oberstdorf	Gerasimova & Beck M-0290433 (M)	<b>MT169979</b>	<b>MT180449</b>	<b>MT162223</b>
<b>153</b>	<i>T. dissimilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	Urbanavichene & Urbanavichus L-15293 (LE)	<b>MT169982</b>	<b>MT180452</b>	<b>MT162226</b>
<b>154</b>	<i>T. dissimilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	Urbanavichene & Urbanavichus L-15294 (LE)	<b>MT169983</b>	<b>MT180453</b>	<b>MT162227</b>
<b>158</b>	<i>T. dissimilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	Urbanavichene & Urbanavichus L-15297 (LE)	<b>MT169985</b>	<b>MT180455</b>	<b>MT162229</b>
<b>159</b>	<i>T. dissimilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	Urbanavichene & Urbanavichus L-15298 (LE)	–	<b>MT180456</b>	–
<b>161</b>	<i>T. dissimilis</i>	Russia	Abkhazia, Ritsinsky Relic National Park	Gerasimova L-11665 (LE)	–	<b>MT180458</b>	<b>MT162231</b>
	<i>T. illudens</i>	Canada	no indication	Westberg TNW2182	MG926037	MG926301	MG925943
	<i>T. separabilis</i>	Sweden	no indication	Ekman 3413	AF282125	MG926236	MG925851
	<i>T. separabilis</i>	Norway	Ostfold, Marker	O-L-105331	MG838165	–	–
	<i>T. separabilis</i>	Norway	Oslo, Oppsal	O-L-200148	MG838175	–	–
	<i>T. separabilis</i>	Norway	Nordland, Hamaroy	O-L-206520	MG838176	–	–
	<i>T. separabilis</i>	Norway	Sogn og Fjordane, Laerdal	O-L-197862	MG838186	–	–
	<i>T. separabilis</i>	Switzerland	no indication		KX098342	–	–
<b>053</b>	<i>T. separabilis</i>	Russia	Murmansk Region, Pasvik Nature Reserve	Urbanavichus M-0182602 (M)	<b>MT169969</b>	<b>MT180439</b>	<b>MT162213</b>
<b>073</b>	<i>T. separabilis</i>	Russia	Kaliningrad Region, Curonian Spit	Gerasimova M-0182613 (M)	<b>MT169970</b>	<b>MT180440</b>	<b>MT162214</b>
<b>110</b>	<i>T. separabilis</i>	Russia	Altay Region, Cherginsky Range	Davydov M-0289891 (M)	<b>MT169971</b>	<b>MT180441</b>	<b>MT162215</b>
<b>118</b>	<i>T. separabilis</i>	Estonia	Valgamaa	Lõhmus TU81156 (TU)	<b>MT169972</b>	<b>MT180442</b>	<b>MT162216</b>
<b>119</b>	<i>T. separabilis</i>	Estonia	Harjumaa	Suja TU76769 (TU)	<b>MT169973</b>	<b>MT180443</b>	<b>MT162217</b>
<b>145</b>	<i>T. separabilis</i>	Germany	Oberallgäu, Bad Hindelang	Gerasimova & Beck M-0290425 (M)	<b>MT169974</b>	<b>MT180444</b>	<b>MT162218</b>
<b>146</b>	<i>T. separabilis</i>	Germany	Oberallgäu, Bad Hindelang	Gerasimova & Beck M-0290425 (M)	<b>MT169975</b>	<b>MT180445</b>	<b>MT162219</b>
<b>147</b>	<i>T. separabilis</i>	Germany	Oberallgäu, Markt Oberstdorf	Gerasimova & Beck M-0290430 (M)	<b>MT169976</b>	<b>MT180446</b>	<b>MT162220</b>

(Continued)

**Table 1.** (Continued)

DNA no. (JG)	Name	Country	Locality	Specimen voucher	GenBank Accession number		
					nrITS	RPB2	mtSSU
<b>151</b>	<i>T. separabilis</i>	Germany	Oberallgäu, Markt Oberstdorf	<i>Gerasimova &amp; Beck</i> M-0290436 (M)	<b>MT169980</b>	<b>MT180450</b>	<b>MT162224</b>
<b>152</b>	<i>T. separabilis</i>	Germany	Oberallgäu, Markt Oberstdorf	<i>Gerasimova &amp; Beck</i> M-0290437 (M)	<b>MT169981</b>	<b>MT180451</b>	<b>MT162225</b>
<b>155</b>	<i>T. separabilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	<i>Urbanavichene &amp; Urbanavichus</i> L-15295 (LE)	<b>MT169984</b>	<b>MT180454</b>	<b>MT162228</b>
<b>160</b>	<i>T. separabilis</i>	Russia	Republic of Adygea, Caucasus Biosphere Reserve	<i>Urbanavichus &amp; Urbanavichene</i> L-15299 (LE)	<b>MT169986</b>	<b>MT180457</b>	<b>MT162230</b>

Maximum likelihood (ML) analysis was performed with RAxML v.8.2.4 following a GTRGAMMA model of molecular evolution with bipartitions drawn onto the most likely tree topology using a multiple non-parametric bootstrap (Stamatakis 2014) on the CIPRES web portal (Miller *et al.* 2010).

Further tree reconstruction using ML analysis was performed in IQ-TREE v.1.6.12 with ultrafast bootstrap approximation (Nguyen *et al.* 2015; Hoang *et al.* 2017). Ultrafast bootstrap (UFBoot) was specified with 10 000 replicates, 1000 maximum iterations and 0.99 minimum correlation coefficient (Minh *et al.* 2013). Substitution models for concatenated and single-locus datasets were selected using ModelFinder (Kalyanamoorthy *et al.* 2017). The TIM2e + I + G4 substitution model was selected for the 3-locus dataset, HKY + F + G4 for the nrITS and mtSSU datasets, and TIM2e + G4 for the RPB2 dataset.

The ML trees based on the different substitution models from the single and concatenated datasets were congruent and in accordance with the Bayesian tree topology. Therefore, only the RAxML tree for the concatenated dataset is shown, with bootstrap support values (BS), posterior probabilities (PP) and ultrafast bootstrap values (UFBoot) added above or below branches. The phylogenetic trees were visualized using FigTree v.1.4.2 (Rambaut 2009). Only clades with BS  $\geq$  70% in RAxML, PP  $\geq$  0.95 in BI and UFBoot  $\geq$  85% in IQ-TREE were considered highly supported and are indicated in bold. The concatenated and individual gene trees obtained from RAxML, MrBayes and IQ-TREE are provided in Supplementary Material File S1 (Figs S1–S13).

### Species delimitation analyses

Molecular and morphological results were complemented with three widely used species delimitation computational approaches: 1) Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2011); 2) Poisson Tree Processes (PTP) (Zhang *et al.* 2013); 3) the Generalized Mixed Yule coalescent approach (GMYC) (Pons *et al.* 2006). ABGD accounts for the barcoding gap (i.e., the difference between intraspecific and interspecific genetic distances) and results can be compared to morphological, geographical or ecological data (Puillandre *et al.* 2011). We carried out ABGD using the online server at <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>, applying defaults following the recommendation of Puillandre *et al.* (2011). One of the most critical parameters of the ABGD method is the prior maximum divergence of intraspecific diversity (P). We assigned the range of Pmin and Pmax to 0.001 and 0.1 (defaults) but focused on

the result for P = 0.01 as previous analyses have demonstrated that the method works best for this value (Puillandre *et al.* 2011).

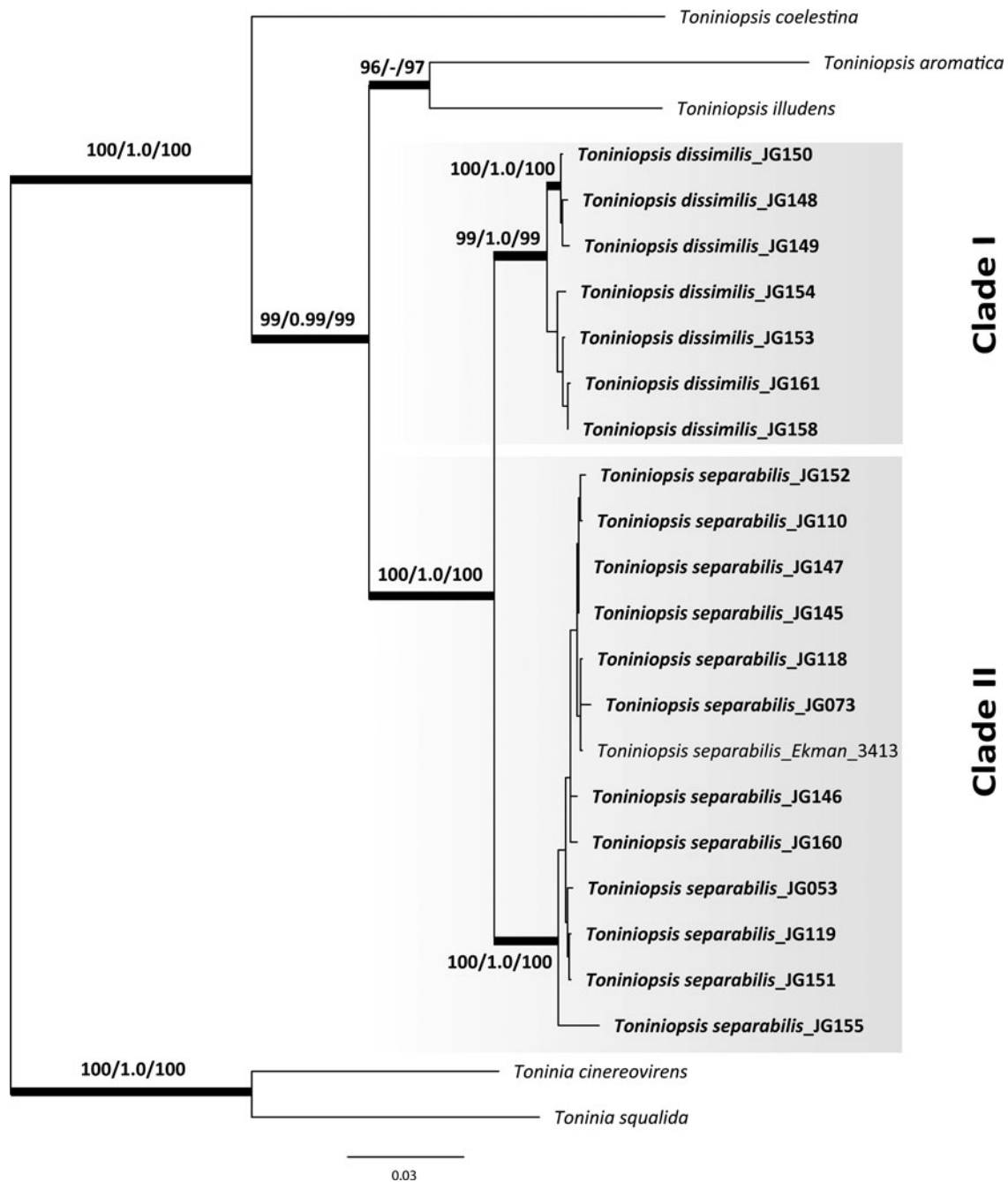
PTP was carried out on the online server (<https://species.h-its.org/ptp/>) using 100 000 MCMC generations (as recommended for small trees with < 50 taxa), saving every 100th generation with 10% discarded as burn-in. PTP is a model for delimiting species based on a phylogenetic tree. Thus, the fully resolved, optimal tree obtained from the 3-locus concatenated data from RAxML was used as an input file, but with the outgroup excluded because this can improve delimitation results (Zhang *et al.* 2013).

A close relative of PTP is the GMYC model but the latter requires an ultrametric rooted tree as input with no zero branch lengths (Fujisawa & Barraclough 2013). To that end, identical sequences were excluded and an ultrametric tree was generated using BEAST v.1.10.4 (<https://beast.community/index.html>). We used BEAUti v.1.10.4 (Drummond *et al.* 2012), an interactive graphical application included in the BEAST package, to generate an xml file using the following parameters: GTR + I + G model as selected by jModelTest; estimated base frequencies; a strict clock model (assumes that all branches on the tree have the same rate of evolution); Tree Prior (Speciation: Yule Process model; best suited to study the relationships between species) with a Random starting tree option (Yule 1925; Gernhard 2008), and adjusting the priors in accordance with parameters received after model calculation (parameters and table with details on prior distributions are given in Supplementary Material File S2, available online). A run of 1 M iterations logging every 100th iteration was conducted. The convergence of the Markov chain was checked using Tracer v.1.7.1. A consensus tree was generated with TreeAnnotator v.1.8.2 after discarding the initial 10% of trees as burn-in. The output file was converted into a newick file using FigTree v.1.4.2 (Rambaut 2009). GMYC was executed with the gmyc function in the SPLITS package in R (v.2.10; [www.cran.r-project.org](http://www.cran.r-project.org)), employing the single (GMYCs) threshold method following parameters in the SPLITS package manual (Supplementary Material File S2). We applied the single-threshold version of GMYC since it has been shown to outperform the multiple-threshold version (Fujisawa & Barraclough 2013; Talavera *et al.* 2013; Luo *et al.* 2018).

## Results

### Phylogenetic analyses

Eighteen new nrITS, 19 mtSSU and 20 RPB2 sequences of *Toniniopsis subincompta* s. lat. were obtained (Table 1). The ML and BI analyses



**Fig. 1.** Maximum likelihood (ML) tree of *Toniniopsis subincompta* s. lat. resulting from the RAxML analysis (Stamatakis 2014) of a concatenated 3-locus dataset (nrITS, mtSSU and *RPB2*). Maximum likelihood bootstrap values (BS), Bayesian posterior probabilities (PP) and ultrafast bootstrap values (UFB) are shown above or below branches. Branches with BS  $\geq 70\%$  in the RAxML analysis (first value), PP  $\geq 0.95$  (second value) and UFB support  $\geq 85\%$  are considered highly supported and marked in bold.

recovered highly concordant topologies in the phylogenetic trees from single genes separately and the concatenated dataset, with *Tonia cinereovirens* (Schaer.) A. Massal. and *T. squalida* (Ach.) A. Massal. used as outgroup (outgroup selection based on Kistenich *et al.* (2018)). Our phylogenetic results using three loci depict two distinct clades in *Toniniopsis subincompta* s. lat. (Fig. 1; Supplementary Material File S1 (Fig. S1), available online). Both clades were recovered with high support (BS/PP/UFB: 99/1.0/99 and 100/1.0/100, respectively). Clade I contains a highly supported subclade including JG148, 149 and 150 (BS/BI/UFB: 100/1.0/100).

Clade I (*T. dissimilis*) comprises specimens from the mountain region of Allgäu and the North Caucasus collected at elevations from 1050 to 1900 m a.s.l., and Clade II (*T. separabilis*) contains those from the Allgäu and North Caucasus in addition to those from Estonia and other parts of Russia (Altay, Murmansk and Kaliningrad regions) collected at elevations from 4 to 1800 m a.s.l. When including six nrITS sequences of *T. subincompta* from GenBank collected in Switzerland (Mark *et al.* 2016) and Norway (Ekman 2001; Kistenich *et al.* 2018), it was shown that they mostly belong to Clade II, except MG838157 in Clade I,

still with high support values for both clades (BS/BI/UFBoot: 99/1.0/100 and 98/1.0/95, respectively; see Figs S2–S4 in Supplementary Material File S1). Taken together, we observed no clear correlation between the clades and geography in our limited dataset. Nevertheless, individuals in Clade I have so far been collected only above 1000 m or in high latitudes (Finnmark, Norway) and thus seem to be more cold-adapted than specimens in Clade II.

### Species delimitation

Molecular species delimitation programs belong to the methods within an integrative taxonomic framework and do not require a prior hypothesis of putative number of species. Species estimates for *T. subincompta* s. lat. using ABGD, PTP and GYMC resulted in two main species clusters in accordance with the results received from phylogenetic analyses. Thus, the first cluster included individuals from Clade I of the phylogenetic tree, and the second cluster individuals from Clade II.

### Morphology

We examined the morphology and anatomy of all specimens of *T. subincompta* s. lat. included in our phylogeny, as well as relevant type specimens and herbarium material available in M (55 specimens). When comparing specimens from the two clades, we did not observe a strong difference in the size of the hymenium, exciple and ascospores (Table 2). The hymenium in Clade I is slightly thicker when compared to Clade II ( $72.5 \pm 17$  and  $66.1 \pm 11.1$   $\mu\text{m}$ , respectively). Ascospores in both clades are predominantly bacilliform, with 1 to 7 septa:  $14\text{--}44 \times 2\text{--}4$   $\mu\text{m}$  in Clade I, and  $13\text{--}40 \times 2\text{--}4$   $\mu\text{m}$  in Clade II. However, differences in thallus structure, as well as apothecia, exciple and hypothecium colour were observed (Table 2). Specimens in Clade I are characterized by having a predominantly wrinkled, warted to subsquamulose thallus whereas in Clade II the thallus mostly consists of single or contiguous  $\pm$ loose granules, often forming short, coralloid, isidium-like bulges (Fig. 2). Apothecia in Clade I are generally grey-brown to dark brown, often with a lighter margin, whereas in Clade II apothecia are mainly darker, dark brown to black, with a margin mostly of the same colour or darker than the disc (pale brown in the albino morph). The hypothecium in Clade I is rather thick, often gradually merging into the coloration of the exciple downwards, whereas in Clade II the hypothecium is thinner and can easily be separated from the exciple below (Fig. 3). While in Clade I the rim of the exciple is mostly colourless with 2–3 layers of enlarged lumina cells (Figs 3A–E, 4A), in Clade II the rim and lateral part often contain either blue-green or brown or mixed blue-brown coloration in the upper part or along the whole margin (Fig. 3B, D & F), with mainly one layer of enlarged lumina cells (Fig. 4B). Two isolates collected from the same tree, representing the dark morph (JG145) and the albino morph (JG146) and confirmed to be the same species, nested in Clade II, but differed from other sequences by at least one nucleotide change in all three genes. In detail, the albino morph differs from the dark morph (JG145) by 4 nucleotides in nrITS, 2 nucleotides in mtSSU and one nucleotide in RPB2. Cyanotrophy, facultative or obligate association of lichens to free-living or  $\pm$ lichenized cyanobacteria (Poelt & Mayrhofer 1988), and the occurrence of an albino morph were observed only in Clade II (see further details below). Based on the morphological and phylogenetic analyses,

we conclude that there are two different species within *Toniniopsis subincompta* s. lat.

### Taxonomic treatment

The name *Lecidea subincompta* Nyl. was introduced as *nomen novum* for *Lecidea anomala* var. *atrosanguinea* Schaer. (Nylander 1865), because the epithet *atrosanguinea* was blocked by *Lecidea atrosanguinea* (Hoffm.) Nyl. (Nylander 1854) which was already in use. *Lecidea anomala* var. *atrosanguinea* Schaer. was based on Schaerer's *Lichenes Helvetici* exs. 212, which in fact contains specimens of *Bacidia incompta* (Borrer ex Hook) Anzi (Ekman 1996). This agrees with our observations on the type material in M (M-0308484). Hence *Toniniopsis subincompta*, based on *Lecidea subincompta*, would have to be regarded as a synonym of *Bellicidia incompta* (Borrer) Kistenich *et al.* (formerly *Bacidia incompta*). For this reason, Ekman (1996) suggested it was appropriate to select a new, conserved type specimen for *Lecidea subincompta* since the epithet *subincompta* was well known, while *Bacidia separabilis* (Nyl.) Arnold, the name to be used without a conserved type, had hardly ever been used. The necessity of conservation was also stated by Kistenich *et al.* (2018) when the taxon was transferred to *Toniniopsis*, but a formal proposal has never been made. According to the research presented here, the former *T. subincompta* consists of two species, and therefore a proposal to conserve *L. subincompta* (with a conserved type) will most likely not be approved. Consequently, *T. subincompta* is synonymized here with *Bellicidia incompta*. While working on the type material of *T. subincompta* s. lat., we did not find a matching candidate for Clade I and thus a new species must be described.

### *Toniniopsis dissimilis* Gerasimova & A. Beck sp. nov. [Clade I]

Mycobank No.: MB 836224

This species is similar to *Toniniopsis separabilis* but differs in having a smooth to rather thick, tuberculate, warted or subsquamulose thallus and dark brown hypothecium, merging into the coloration of the exciple downwards.

Type: Germany, Bavaria, Landkreis Oberallgäu, Markt Oberstdorf, track from Oytalhaus to Käseralpe, c. 500 m east of Oytalhaus, mixed forest of *Picea*, *Fraxinus* and *Salix* along the River Oybach, on bark of the trunk of *Fraxinus*, c. 1 m above the ground, J. Gerasimova & A. Beck s. n., 27 June 2018 (M-0290432—holotype; UPS—isotype).

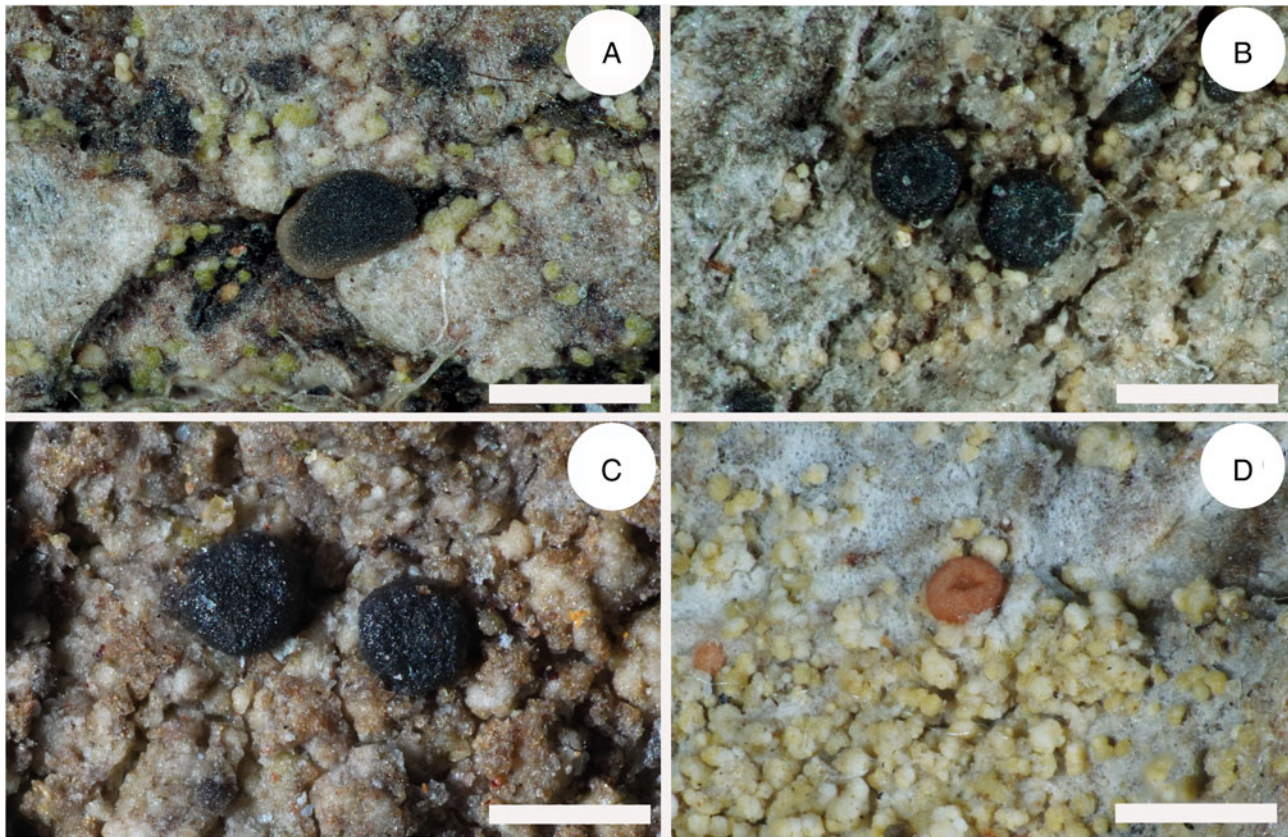
(Figs 2A & C, 3A, C & E, 4A)

*Thallus* indeterminate, thin, partly smooth to rather thick, tuberculate or warted, sometimes consisting of an appressed and continuous crust of subsquamules, granular when on twigs of mosses, consisting of single or contiguous warts or occasionally of single  $\pm$ rounded granules. *Warts*  $\pm$ flattened or forming subsquamules, adnate to the surface, often merged, forming a thick, wrinkled, irregularly shaped surface, light green, grey-green, green, brown-green, dirty grey-green to green-brown. *Photobiont* chlorococcoid.

*Apothecia* (0.2–)0.4  $\pm$  0.15(–0.7) mm diam. ( $n_1 = 77$ ,  $n_2 = 8$ ), young with margin raised above the disc, mature  $\pm$ flat, later becoming convex. *Disc* grey-brown, dark brown to black, sometimes with mixed colour. *Margin* of the same colour as or paler than disc, light brown, grey, brown-grey, grey-brown to dark

**Table 2.** Comparison of main diagnostic features between specimens from Clade I and Clade II of *Toniniopsis subincompta* s. lat.

	Clade I	Clade II
Thallus structure	Partly smooth, mostly tuberculate or warted sometimes consists of appressed and continuous crust of subsquamules or scattered or contiguous warts	Partly smooth, mostly consists of scattered $\pm$ roundish granules, often forming short, coralloid, isidium-like bulges; sometimes associates with cyanobacteria, thus forming dark green or black crust or bulges
Apothecia (colour)	Greyish brown, dark brown to black; margin brighter or concolorous, often lighter than disc	Grey-brown, dark brown to black or orange (albino morph); margin concolorous or darker (can be lighter brown); albino morph often presented
Epithecium	Colourless to brown, grey-blue, blue or blue-green	Brown to grey-blue to blue-green
Hypothecium	Often gradually merging into the coloration of the exciple downwards	Can be separated from the exciple below
Hymenium ( $\mu\text{m}$ )	(51–)72.5 $\pm$ 17(–122.5)	(49–)66.1 $\pm$ 11.1(–98)
Exciple ( $\mu\text{m}$ )	(30–)63 $\pm$ 19.3(–115)	(59–)63.5 $\pm$ 18(–122.5)
Rim of exciple	Mostly colourless or blue	Brown, blue or mixed
Lateral exciple	Brown	Brown, blue or mixed
Lumina cells along exciple rim, size ( $\mu\text{m}$ )	2–3 layers (2–)4.2 $\pm$ 1.2(–7) $\times$ (5–)8.35 $\pm$ 2.9(–24)	1–2 layers (2.0–)3.5 $\pm$ 0.8(–6) $\times$ (5–)7.5 $\pm$ 1.8(–13)
Spores: size ( $\mu\text{m}$ )	(14–)26.7 $\pm$ 4.2(–44) $\times$ (2–)2.9 $\pm$ 0.4(–4)	(13–)25.2 $\pm$ 4.2(–40) $\times$ (2–)2.7 $\pm$ 0.42(–4)
Spores: septa	(1–)4 $\pm$ 2(–7)	(1–)4 $\pm$ 2(–7)



**Fig. 2.** Detail of *Toniniopsis dissimilis* and *T. separabilis*. A, holotype of *T. dissimilis* (M-0290432, JG149); thallus, consisting of scattered ±rounded or flattened or subsquamulose granules, and dark apothecia with light brown margin. B, *T. separabilis* (LE L-15299, JG160); thallus thin, consisting of ±rounded, loose granules. C, *T. dissimilis* (LE L-15293, JG153); thallus thick, warty to wrinkled. D, *T. separabilis* (M-0182613, JG073); thallus thick, wrinkled, forming isidium-like bulges with albino morph apothecia. Scales: A–D = 0.5 mm. In colour online.

brown. *Hymenium* (51–)72.5 ± 17(–122.5) µm thick ( $n_1 = 35$ ,  $n_2 = 8$ ), colourless, without crystals. *Epithecium* greyish green or greyish blue, green-blue, partly almost colourless or brown. *Hypothecium* brown to dark brown, rather thick, downwards gradually merging into the coloration of the exciple. *Exciple* (30–)63 ± 19.3(–115) µm wide ( $n_1 = 34$ ,  $n_2 = 8$ ), sometimes with minute crystals, not dissolving in K. *Rim* colourless, without or with greyish blue or blue tinge near the hymenium or along the whole margin, with 2–3 layers of cells with enlarged lumina (2–)4.2 ± 1.2(–7) µm wide ( $n_1 = 40$ ,  $n_2 = 8$ ), and (5–)8.3 ± 2.9(–24) µm long ( $n_1 = 40$ ,  $n_2 = 8$ ; maximum value with 24 µm was observed only once in JG149). *Lateral part* colourless or pale brown to dark brown, often coloured mainly when attached to hymenium. *Medullary part* pale brown, brown to almost colourless downwards, paler than hypothecium. *Paraphyses* simple or septate, (1.5–)1.8 ± 0.4(–3.5) µm wide ( $n_1 = 42$ ,  $n_2 = 8$ ); apices ±clavate or not at all swollen, sometimes bifurcate, (1.5–)2.5 ± 0.7(–4) µm wide ( $n_1 = 42$ ,  $n_2 = 8$ ), colourless or with dark blue diffuse internal pigmentation. *Ascospores* bacilliform, (14–)26.7 ± 4.2(–44) µm long ( $n_1 = 245$ ,  $n_2 = 8$ ), and (2–)2.9 ± 0.4(–4) µm wide ( $n_1 = 245$ ,  $n_2 = 8$ ), with (1–)4 ± 2(–7) septa ( $n_1 = 245$ ,  $n_2 = 8$ ).

**Chemistry.** Hypothecium K+ intense brown to purplish; exciple K+ purplish brown.

**Pigments.** Bagliettoa-green in epithecium and in uppermost part and rim of exciple; Laurocerasi-brown in hypothecium and lateral part of exciple.

**Etymology.** Similar to *T. separabilis* but differs in some characters.

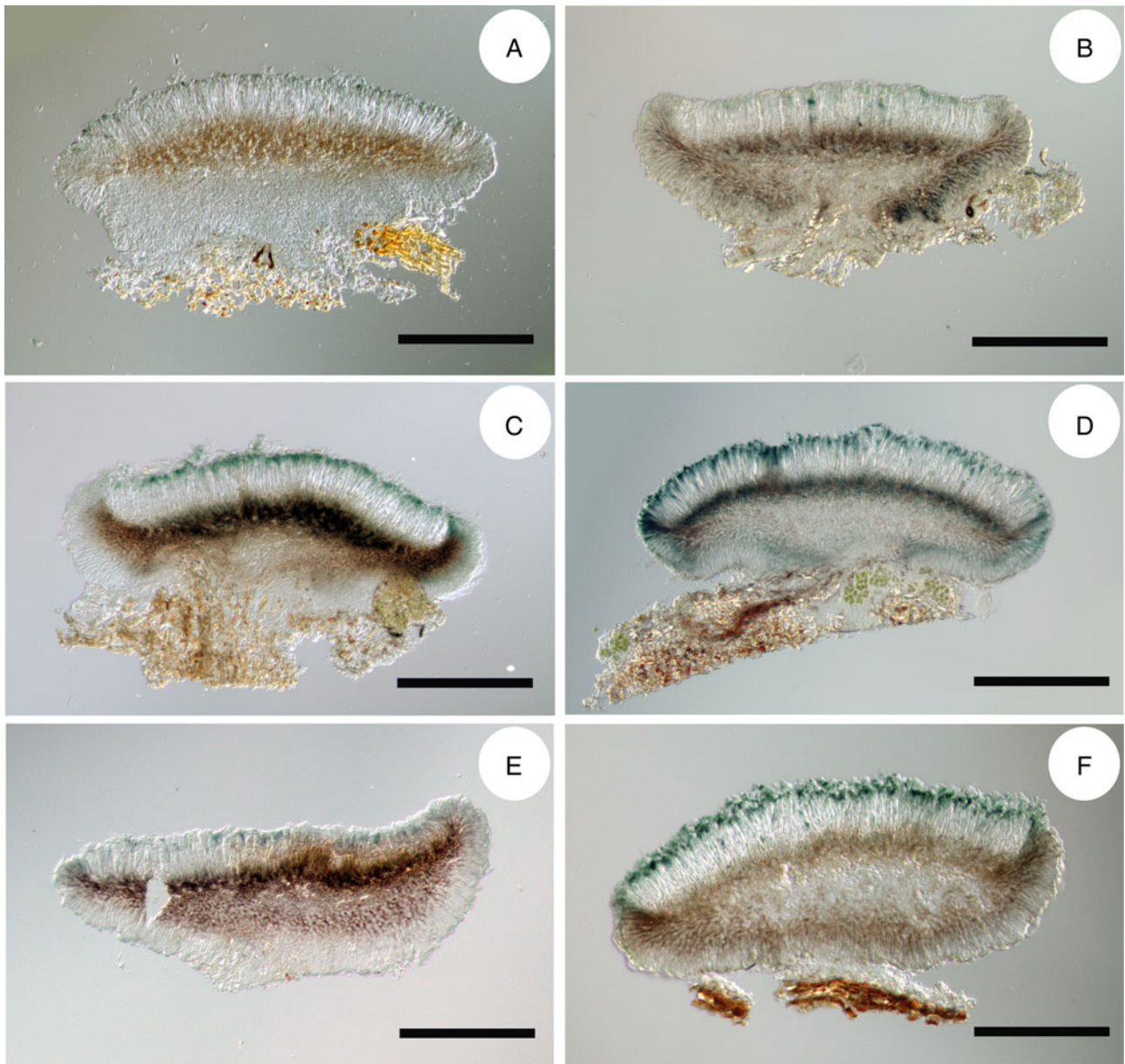
**Additional specimens examined.** **Turkey:** Prov. Rize: valley very near to the village of Ayder, 30 vii 1997, V. John (M-0308422). Prov. Bursa: Uludag, 6 ix 1976, K. Kalb & G. Ploebst (M-0308424).—**Germany:** Bayerische Alpen: am Taubensee ober Wessen, ix 1876, F. Arnold (M-0308441); *ibid.*, ix 1871, F. Arnold (M-0308442); Aschau, ix 1875, F. Arnold (M-0308443).—**Sweden:** Södermanland: Björkvik, 27 vi 1908, G. O. A. Malme (M-0308448).—**Norway:** Troms: Tromsø, 1868, Th. M. Fries (M-0308451).—**Finland:** [South Häme]: Luhanka, 1873, E. Lang (M-0308456).—**Slovakia:** Malá Fatra: Velký Stoh, c. 1300 m, 17 vi 1965, I. Pišút & A. Vězda (M-0308458).—**Austria:** Steiermark: Niedere Tauern, Wölzer Tauern, 1500 m, 15 vii 1990, J. Hafellner (M-0308468). Vorarlberg: Rätikon, Gargellen, 1300 m, 6 ix 1989, R. Türk (M-0308469). Tirol: Matrei, F. Arnold (M-0308476, M-0308477).

*Toniniopsis separabilis* (Nyl.) Gerasimova & A. Beck comb. nov. [Clade II]

Mycobank No.: MB 836225

*Lecidea separabilis* Nyl., *Flora (Jena)* 48(10), 147 (1865).—*Bacidia atrosanguinea* f. *separabilis* (Nyl.) Arnold, *Flora (Jena)* 67(30), 582 (1884); type: [Finland, South Häme], Hollola, 1863,





**Fig. 3.** Transverse sections of apothecia of *Toniniopsis dissimilis* (A, C, E) and *T. separabilis* (B, D, F). A, holotype of *T. dissimilis* (M-0290432, JG149). B, lectotype of *Lecidea separabilis* (H-NYL 17424). C, colourless rim of exciple; lateral part brown, mainly attached to hymenium, partly with blue tinge near hymenium (M-0290431, JG148). D, rim and lateral part of exciple with blue coloration along the whole margin (M-0290425, JG145; dark morph). E, dark brown hypothecium, merging into coloration of exciple below (L-15293, JG153). F, dark brown hypothecium with paler exciple below; rim and lateral part of exciple with brown coloration along the whole margin (M-0290437, JG152). Scales: A–F = 200  $\mu$ m. In colour online.

*J. P. Norrlin* s. n. (H-NYL 17424—lectotype! selected by Ekman (1996: 105)).

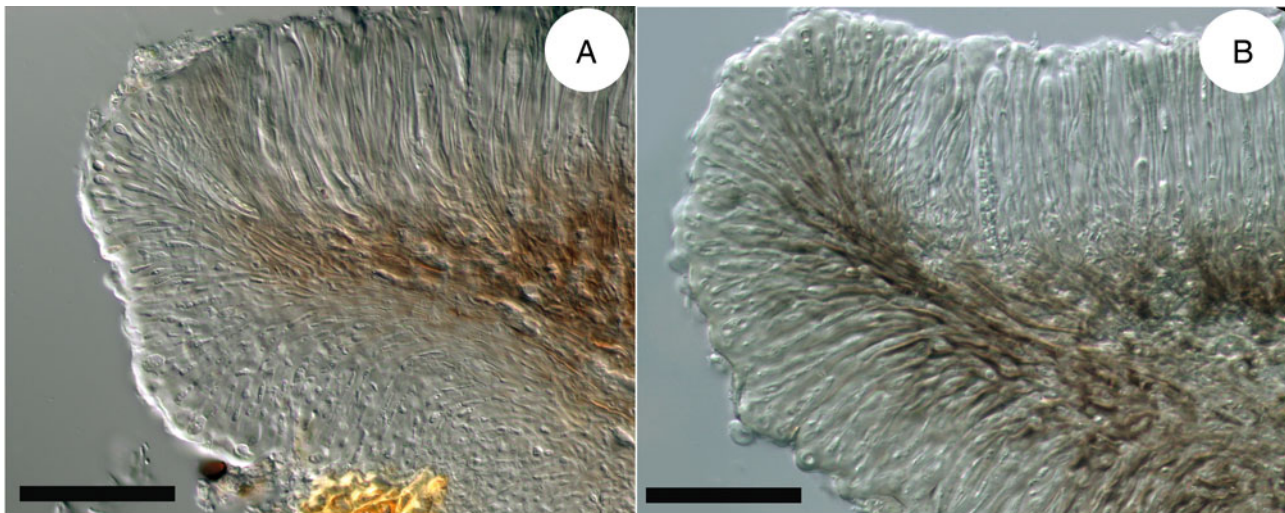
*Lecidea hegetschweileri* Hepp [nom. nud.] in *Hepp's Systematische Sammlung*, 212 (1852).—*Biatora atrosanguinea* [unranked] *hegetschweileri* Hepp, *Die Flechten Europas* 1 (1853).—*Bacidia hegetschweileri* (Hepp) Vain. [non auct.], *Acta Soc. Fauna Flora Fenn.* 53(1), 215 (1922); type: [Switzerland], Zürich, An der Rinde alter Eichen, *J. A. P. Hepp* s. n. [Hepp: *Flecht. Europ.*, 23] (BM00002217—lectotype! selected by Ekman (1996: 105); M-0190116, M-0154468—isolectotypes!).

*Secoliga atrosanguinea* var. *affinis* Stizenb., *Nova Acta Acad. Leopoldin.-Carolin.* 30(3), 18 (1863) [non *Bacidia atrosanguinea* (Sch.) var. *affinis* Zw. in Arnold: *Lich. exs.* 505].—*Bacidia affinis*

(Stizenb.) Vain., *Acta Soc. Fauna Flora Fenn.* 53(1), 154 (1922); type: [Germany, Baden-Württemberg], Heidelberg, in der Nähe des Kohlhofs, an *Popul. tremula*, sehr selten, 1858, *Zwackh-Holzhausen* s. n. [*Lichenes exs.* 336B] (H-NYL 17397—lectotype! selected here, MBT394654).

*Lecidea bacillifera* f. *melanotica* Nyl., *Flora (Regensburg)* 50, 373 (1867).—*Bacidia subincompta* var. *melanotica* (Nyl.) H. Magn., *Förteckning över Skandinavians växter* 4, 40 (1936); type: Finland, [South Häme], Evo (= Evo), ad corticem populi, 1866, *J. P. Norrlin* s. n. (H-NYL 17205—lectotype! selected here, MBT394655; H-NYL 17362—isolectotype!).

*Bacidia vegeta* Vain., *Acta Soc. Fauna Flora Fenn.* 53(1), 153 (1922); type: [Finland, South Häme], Mustiala, 1868, A.



**Fig. 4.** Exciple structure. A, holotype of *Toniniopsis dissimilis* (M-0290432, JG149) with at least 3 layers of enlarged lumina cells. B, lectotype of *Lecidea separabilis* (H-NYL 17424) with paraplectenchymatic exciple structure and one layer of enlarged lumina cells. Scales: A & B = 50  $\mu$ m. In colour online.

*Kullhem* s. n. (H8000177—lectotype! annotated by S. Ekman (2012), selected here, MBT394656).

(Figs 2B & D, 3B, D & F, 4B, 5)

*Thallus* indeterminate, thin, partly smooth, inconspicuous, discrete to rather thick, continuous, warted, thinly to coarsely granular, consisting of single or contiguous  $\pm$  loose granules, especially rich in cracks of the bark, often forming short, coralloid, isidium-like bulges. *Granules*  $\pm$  roundish or flattened or forming patches of irregularly shaped subsquamules, green, green-grey to green-brown, turning completely brown in the herbarium. *Photobiont* chlorococcoid, sometimes additionally associating with one or several free-living cyanobacteria: 1) *Nostoc* sp., 2) *Gloeocapsa* sp. and 3) *Scytonema* sp. (cyanotrophic association).

*Apothecia* (0.2–)0.4  $\pm$  0.1(–0.8) mm diam. ( $n_1 = 88$ ,  $n_2 = 10$ ), young  $\pm$  flat or with margin slightly above the disc, mature  $\pm$  flat, later becoming convex and irregularly shaped. *Disc* grey-brown, dark brown to black or pale orange to orange-brown in albino morphs. *Margin* of the same colour as or darker than the disc, light brown, brown to dark brown to black. *Hymenium* (49–)66.1  $\pm$  11.1(–98)  $\mu$ m thick ( $n_1 = 43$ ,  $n_2 = 10$ ), colourless, without crystals. *Epithecium* brown, greyish blue to dark blue-green or sometimes almost colourless (albino morph). *Hypothecium* thin, brown to dark brown, sometimes with green tinge, or straw-coloured, almost colourless in the albino morph, can be easily separated from exciple below. *Exciple* (59–)63.5  $\pm$  18(–122.5)  $\mu$ m wide ( $n_1 = 39$ ,  $n_2 = 10$ ), without or with minor crystals along exciple, not dissolving in K. *Rim* pale brown, dark brown or blue, often mixed blue-brown, mostly with one layer or up to two layers of enlarged lumina cells along the rim, often inconspicuous, (2.0–)3.5  $\pm$  0.8(–6)  $\mu$ m wide ( $n_1 = 55$ ,  $n_2 = 10$ ) and (5–)7.5  $\pm$  1.8(–13)  $\mu$ m long ( $n_1 = 55$ ,  $n_2 = 10$ ). *Lateral part* pale brown to brown mainly attached to hymenium, often with blue, brown or mixed blue-brown colour near hymenium or along the whole margin, or almost colourless or pale straw-coloured (albino morph). *Medullary part* under hypothecium light brown to almost colourless which downwards can eventually change to dark brown or blue. *Paraphyses* simple, (1–)1.8  $\pm$  0.5(–3.5)  $\mu$ m wide ( $n_1 = 60$ ,  $n_2 = 10$ ); apices  $\pm$  clavate or not at all swollen,

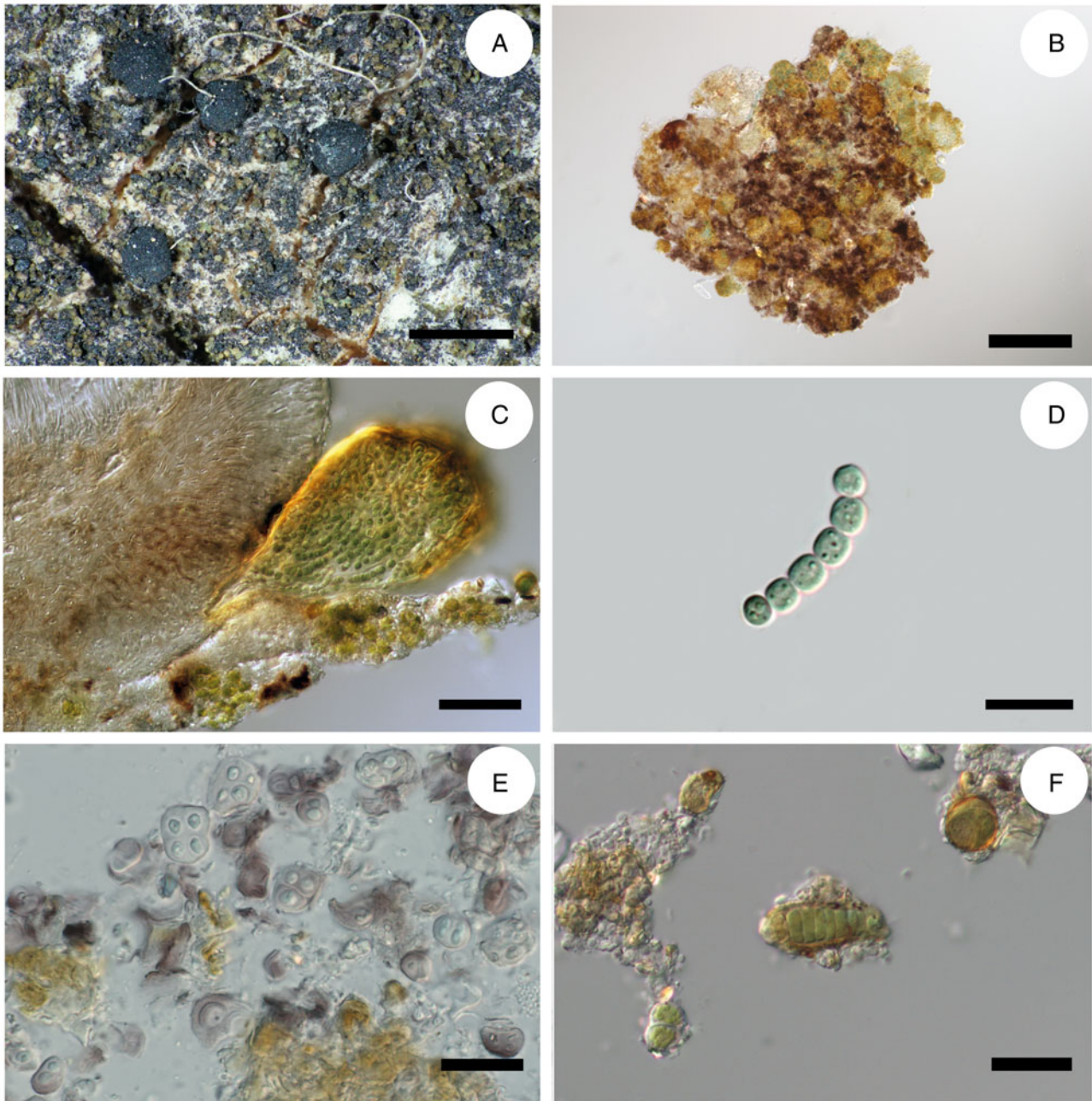
sometimes bifurcate, (1.5–)3  $\pm$  1(–5.0)  $\mu$ m wide ( $n_1 = 60$ ,  $n_2 = 10$ ), colourless or with dark greyish blue to dark blue-green diffuse internal pigmentation. *Ascospores* bacilliform or acicular, (13–)25.2  $\pm$  4.2(–40)  $\mu$ m long ( $n_1 = 273$ ,  $n_2 = 10$ ) and (2–)2.7  $\pm$  0.42(–4)  $\mu$ m wide ( $n_1 = 273$ ,  $n_2 = 10$ ), with (1–)4  $\pm$  2(–7) septa ( $n_1 = 273$ ,  $n_2 = 10$ ).

**Chemistry.** Hypothecium K<sup>+</sup> intense brown; brown parts of exciple K<sup>+</sup> purplish brown.

**Pigments.** Bagliettoa-green in epithecium and rim of exciple; Laurocerasi-brown in hypothecium and lateral part of exciple; sometimes a mixture of Bagliettoa-green and Laurocerasi-brown in exciple. Rubella-orange in epithecium, hypothecium and exciple (in the case of the albino morph).

**Remarks.** According to the International Code of Nomenclature, for any taxon below the rank of genus, the correct name is the combination of the final epithet of the earliest legitimate name of the taxon at the same rank (Art. 11.4, Turland et al. 2018). Therefore, *Lecidea separabilis* has priority at species rank from 1865, whereas the epithets *hegetschweileri* and *affinis* were validly published at the species rank only in 1922 (Vainio 1922). The complex nomenclature regarding the name *Bacidia hegetschweileri* has already been described in detail by Ekman (1996) so we do not reiterate this information here.

**Additional specimens examined.** **Russia:** Republic of Adygea: Maykop district, 1900 m, I. Urbanavichene & G. Urbanavichus (LE L-15296); *ibid.*, 1460 m, I. Urbanavichene & G. Urbanavichus (LE L-15301). **Kabardino-Balkaria:** Elbrus National Park, 1880 m, I. Urbanavichene & G. Urbanavichus (LE L-15302).—**Canada:** Graham Island: Mt Raymond, c. 760 m, 1988, I. M. Brodo 26653B (M-0308421).—**Turkey:** Prov. Kastamonu: Ilgaz Dağları, 1680 m, 11 viii 1976, K. Kalb & G. Ploebst (M-0308423); *ibid.*, 1680 m, 31 viii 1976, K. Kalb (M-0308425).—**Germany:** Landkreis Oberallgäu: Markt Oberstdorf, 850 m, 27 vi 2018, J. Gerasimova & A. Beck (M-0290429); *ibid.*, 1050 m, 27 vi 2018, J. Gerasimova & A. Beck (M-0290434); *ibid.*, 950 m, 29 vi 2018, J. Gerasimova & A. Beck (M-0290439; M-0290440). **Landkreis Berchtesgadener Land:**



**Fig. 5.** Cyanotrophic association in lectotype of *Lecidea bacillifera* f. *melanotica* (A, B & E) and *T. separabilis* (C, D & F). A, thallus with cyanobacterium (dark patches) in tight association. B, composition of the thallus seen under the microscope. C, cross-section of apothecium with associated cyanobacterium cells (M-0289891, JG110). D, *Nostoc* sp. (M-0289891, JG110). E, *Gloeocapsa* sp. F, *Scytonema* sp. (M-0290436, JG151). Scales: A = 1 mm; B = 100  $\mu$ m; C = 50  $\mu$ m; D–F = 15  $\mu$ m. In colour online.

Königssee, 605 m, 1984, R. Türk & H. Wunder 2961 (M-0308431); *ibid.*, 630 m, 1998, R. Türk 25704 (M-0308426); Obersalzberg, 955 m, 2011, R. Cezanne & M. Eichler 8371 (M-0308428); Ramsau, 775 m, 1988, R. Türk & H. Wunder 5988 (M-0308429); Hochbahnweg, 1050 m, 1989, R. Türk & H. Wunder 6257 (M-0308430). *Landkreis Freyung-Grafenau*: National Park Bayerischer Wald, 1100–1180 m, 13 x 1999, Ch. Printzen (M-0308427). *Landkreis Weissenburg-Gunzenhausen*: Weissenburg, 20 v 1866, F. Arnold (M-0308432). *Landkreis München*: zwischen Geiselgasteig und Wörnbrunn, im Grünwalder Park, 3 x 1888, F. Arnold (M-0308434); zwischen Obersending und Hesseloh, vi 1888, F. Arnold (M-0308436). *Landkreis Starnberg*: westlich von

Gauting, 23 iii 1896, F. Arnold (M-0308435). *Landkreis Coburg*: Tal W Hohe Schwemge, NW Oberwohlsbach, 1989, L. Meinunger 17353 (M-0308437). *Landkreis Miesbach*: Alpbach, Tegernsee, 1989, L. Meinunger 17135 (M-0308438). *Landkreis Eichstätt*: Eichstätt, 1858, F. Arnold (M-0308440); *ibid.*, v 1864, F. Arnold (M-0308444); *ibid.*, vii 1869, F. Arnold (M-0308445). *Thüringen*: Kreis Hildburghausen, 1989, L. Meinunger 17004 (M-0308446).—**Austria**: *Salzburg*: Brunnfeld between the villages of Pichl-Auhof and Ort, 605 m, 5 iv 2008, R. Türk & S. Pfleger (M-0308465); Hohe Tauern, Kapruner Tal, 970–1270 m, 27 x 1989, R. Türk (M-0308466). *Kärnten*: Rennweg N von Pörtschach, 600 m, 14 ii 1990, R. Türk (M-0308467). *Vorarlberg*: Lechtaler-Alpen, 1300 m,

10 vii 1986, *H. Mayerhofer, G. Grabherr & R. Türk* (M-0308470). *Steiermark*: Oberfahrenbach, 400 m, 19 iii 1995, *J. Poelt* (M-0308471). *Tirol*: Wildpark Wildbichl, vii 1895, *Schnabl* (M-0308439); Matrei, vii 1869, *F. Arnold* (M-0308472, M-0308473); *ibid.*, viii 1872, *F. Arnold* (M-0308478).—**Sweden**: *Värmland*: Ekshärad, 11 vii 1961, *S. W. Sundell* (M-0308447). *Uppland*: Värmdön, 28 vii 1908, *G. O. A. Malme* (M-0308449). *Jämtland*: Enafors, 26 vii 1910, *G. O. A. Malme* (M-0308450).—**Norway**: *Troms*: Tromsø, 1868, *Th. M. Fries* (M-0308452).—**Finland**: [*North Häme*]: Saarijärvi, Saarikylä, 6 viii 1947, *A. Koskinen* (M-0308453); Mahlu, Syväoja, 5 vii 1948, *A. Koskinen* (M-0308454); Mahlu, Lylymäki, 3 vi 1943, *A. Koskinen* (M-0308455). *South Häme*: Evo, 1874, *J. P. Norrlin* (M-0308457).—**Slovakia**: *Žilina Region*: Králova, Királyhegy hola, Teplička, *H. Lojka* (M-0308460).—**Czech Republic**: *Moravia*: Telč, 'Roštynská obora', 550 m, 26 vi 1965, *A. Vězda* (M-0308459); Carpati, prope pagum Ostravice, 700 m, 24 viii 1964, *A. Vězda* (M-0308464).—**Romania**: *Transylvania*: ad ramulos abietum in regione Aragyey infra alpem Retezat, c. 1500 m, 23 viii 1873, *H. Lojka* (M-0308461); *ibid.*, 9 viii 1873, *H. Lojka* (M-0308462); prope Thermas Herculis in Banatu, 16 ix 1872, *H. Lojka* (M-0308463).

## Discussion

Our results clearly demonstrate the presence of two distinct species within the former *Toniniopsis subincompta*. The results obtained from phylogenetic and morphological analyses were congruent with species estimates for *T. subincompta* s. lat. using ABGD, PTP and GYMC. Based on the material examined, mainly from Eurasia, *T. separabilis* is the more common taxon (76.4%) and has the wider distribution range (Canada, Norway, Sweden, Estonia, Germany, Austria, Czech Republic, Slovakia, Romania, Turkey and Russia). *Toniniopsis dissimilis* is less frequent (23.6%) and specimens were examined from Norway, Sweden, Finland, Germany, Slovakia, Austria, Turkey and Russia. The latter species seems to be more cold-adapted as we have only seen specimens collected above 1000 m or in high latitudes.

According to the literature, the distribution of *T. subincompta* s. lat. extends to Macaronesia, Africa and North America (Ekman 1996; Llop 2007; Coppins & Aptroot 2009). Based on observations of specimens from North America by Ekman (1996), *T. subincompta* has the same variation in thallus structure as we observed in specimens from Eurasia. Apothecia are mainly dark, purple-brown to black, and the exciple rim has a single layer of enlarged globose lumina cells which correspond to Clade II but are smaller in size ( $5 \times 8 \mu\text{m}$ ). In some specimens, bacilliform ascospores were found in young apothecia, whereas acicular ascospores were found in old apothecia; a variation in spore shape may even occur within the same apothecium (Ekman 1996, loc. cit.). We found only bacilliform ascospores in all specimens except two, but looked mainly at middle-aged apothecia. The two exceptions with long acicular ascospores were JG053 (Murmansk Region) and the type specimen of *Lecidea bacillifera* f. *melanotica* (Finland). In addition, ascospores in northern American material are longer and thicker with a larger number of septa ( $19\text{--}64 \times 1.9\text{--}6.2 \mu\text{m}$ , with 3–13 septa). Our observations correspond with those on *T. subincompta* from Great Britain and Spain (ascospores bacilliform,  $20\text{--}36\text{--}40 \times 2.3\text{--}3.5\text{--}4 \mu\text{m}$ , with 3–7 septa and  $18\text{--}40 \times 2\text{--}4 \mu\text{m}$ , with 3–9 septa, respectively (Llop 2007; Coppins & Aptroot 2009). A single herbarium specimen observed from Canada was determined to be *T. separabilis* with


characters corresponding to our description. Potentially, *T. subincompta* s. lat. from North America represents a separate entity to those from Eurasia. However, additional morphological and phylogenetic investigations are required.

In *T. subincompta* s. lat., an albino morph had already been mentioned by Coppins & Aptroot (2009). In order to test if dark and albino morphs belong to the same species, we sequenced both, a dark and an albino morph from bark of the same tree. According to our results, the two isolates belong to the same species but differ from each other in three genes. In fact, none of the sequences of dark morphs were identical to the albino morph, while identical sequences of dark morphs did occur. Further studies are required to find out if the albino morphs form a separate lineage or are formed independently by spontaneous mutation.

Cyanotrophy is defined as facultative or obligate association of lichens to free-living or  $\pm$ lichenized cyanobacteria (Poelt & Mayrhofer 1988). We observed the strongest association with cyanobacteria in one of the type specimens, *Lecidea bacillifera* f. *melanotica* (*Gloeocapsa* sp. and *Nostoc* sp. (Fig. 5A, B & E)), which was assigned to *T. separabilis* in this study based on morphological observation. The strong cyanotrophic association appears to lead to a modification of the *melanotica* thallus, represented by black-coloured patches. Similarly, a strong connection was observed in two further herbarium specimens collected in Sweden (M-0308447 and M-0308450), which were associated with *Nostoc* sp. The most diverse composition of cyanobacteria was observed in JG151 (Oberallgäu), with species from three different genera: *Gloeocapsa*, *Nostoc* and *Scytonema*. Juvenile stages of some green-algal lichens may establish loose cyanotrophic associations with free-living cyanobacteria and/or cyanolichens (Rikkinen 2003). Such associations in *Ramalinaceae* are known in *Thalloidima* A. Massal., most species of which are parasitic on cyanolichens when young or remain parasitic (Kistenich et al. 2018). However, the specimens we analyzed were clearly associated with free-living cyanobacteria and were not cyanolichens. Previously, complementary associations with cyanobacteria in crustose lichens have mainly been described from saxicolous species, probably due to low nutrient levels in the substratum (Poelt & Mayrhofer 1988). However, all specimens with cyanobacteria of *T. separabilis* were collected on tree bark (*Picea* sp., *Populus* sp., *Salix* sp., *Thuja* sp. and *Ulmus* sp.). Cyanotrophy was observed only in *T. separabilis* and is considered to be facultative based on our observations that only 11 out of 68 specimens (including herbarium, type and sequenced specimens) examined were cyanotrophic (c. 16%). Albino morphs, which have been found in only four specimens of *T. separabilis* (c. 6%), were likewise not observed in specimens of *T. dissimilis*. These interesting aspects of lichen biology deserve further study.

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## References

- Beck A and Mayr C (2012) Nitrogen and carbon isotope variability in the green-algal lichen *Xanthoria parietina* and their implications on mycobiont-photobiont interactions. *Ecology and Evolution* **2**, 3132–3144.
- Brackel W von (2019) *Rote Liste und Gesamtartenliste der Flechten (Lichenes), flechtenbewohnenden und flechtenähnlichen Pilze Bayerns. Stand 2019*. Augsburg: Bayerisches Landesamt für Umwelt.
- Coppins BJ and Aptroot A (2009) *Bacidia* De Not. 1846. In Smith CW, Aptroot A, Coppins BJ, Fletcher A, Gilbert OL, James PW and Wolseley PA (eds), *Lichens of Great Britain and Ireland*. London: British Lichen Society, pp. 189–207.
- Darriba D, Taboada GL, Doallo R and Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772.
- Drummond AJ, Suchard MA, Xie D and Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**, 1969–1973.
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.
- Ekman S (1996) The corticolous and lignicolous species of *Bacidia* and *Bacidina* in North America. *Opera Botanica* **127**, 1–148.
- Ekman S (2001) Molecular phylogeny of the *Bacidaceae* (Lecanorales, lichenized Ascomycota). *Mycological Research* **105**, 783–797.
- Fujisawa T and Barraclough TG (2013) Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology* **62**, 707–724.
- Gerasimova JV and Ekman S (2017) Taxonomy and nomenclature of seven names in *Bacidia* (Ramalinaceae, Lecanorales) described from Russia. *Phytotaxa* **316**, 292–296.
- Gerasimova JV, Ezhkin AK and Beck A (2018) Four new species of *Bacidia* s.s. (Ramalinaceae, Lecanorales) in the Russian Far East. *Lichenologist* **50**, 603–625.
- Gernhard T (2008) The conditioned reconstructed process. *Journal of Theoretical Biology* **253**, 769–778.
- Gilbert O (1996) The occurrence of lichens with albino fruit bodies (Ascomata) and their taxonomic significance. *Lichenologist* **28**, 94–97.
- Hoang DT, Chernomor O, von Haeseler A, Minh BQ and Le SV (2017) [2018] UFBboot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution* **35**, 518–522.
- Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A and Jermin LS (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* **14**, 587–589.
- Kistenich S, Tindal E, Bendiksby M and Ekman S (2018) Molecular systematics and character evolution in the lichen family Ramalinaceae (Ascomycota: Lecanorales). *Taxon* **67**, 871–904.
- Liu YJ, Whelen S and Hall BD (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**, 1799–1808.
- Llop E (2007) *Lecanorales: Bacidaceae: Bacidia* y *Bacidina*. *Flora Liqueológica Ibérica* **3**, 1–49.
- Luo A, Ling C, Ho SYW and Zhu CD (2018) Comparison of methods for molecular species delimitation across a range of speciation scenarios. *Systematic Biology* **67**, 830–846.
- Mark K, Cornejo C, Keller C, Flück D and Scheidegger C (2016) Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation. *Genome* **59**, 685–704.
- Meyer B and Printzen C (2000) Proposal for a standardized nomenclature and characterization of insoluble lichen pigments. *Lichenologist* **32**, 571–583.
- Miller MA, Pfeiffer W and Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *Proceedings of the Gateway Computing Environments Workshop (GCE) 14 November 2010, New Orleans, Louisiana*, pp. 1–8.
- Minh BQ, Nguyen MAT and von Haeseler A (2013) Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* **30**, 1188–1195.
- Nguyen LT, Schmidt HA, von Haeseler A and Minh BQ (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution* **32**, 268–274.
- Nylander W (1854) Études sur les lichens d'Algérie. *Mémoires de la Société des Sciences Naturelles de Cherbourg* **2**, 305–344.
- Nylander W (1865) Lecideae adhuc quaedam europeae novae. *Flora (Regensburg)* **48**, 145–148.
- Poelt J and Mayrhofer H (1988) Über Cyanotrophie bei Flechten. *Plant Systematics and Evolution* **158**, 265–281.
- Pons J, Barraclough TG, Gómez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sullin WD and Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55**, 595–609.
- Puillandre N, Lambert A, Brouillet S and Achaz G (2011) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21**, 1864–1877.
- Rambaut A (2009) *FigTree v.1.3.1*. [WWW resource] URL <http://tree.bio.ed.ac.uk/software/figtree/>.
- Rikkinen J (2003) Ecological and evolutionary role of photobiont-mediated guilds in lichens. *Symbiosis* **34**, 99–110.
- Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA and Ronquist JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.
- Rose F and Coppins S (2002) Site assessment of epiphytic habitats using lichen indices. In Nimis PL, Scheidegger C and Wolseley PA (eds), *Monitoring with Lichens – Monitoring Lichens*. Dordrecht: Kluwer Academic Publishers, pp. 343–348.
- Rücker T and Wittmann H (1995) Mykologisch-lichenologische Untersuchungen im Naturwaldreservat Kesselfall (Salzburg, Österreich) als Diskussionsbeitrag für Kryptogamenschutzkonzepte in Waldökosystemen. *Beihefte zur Sydowia* **10**, 168–191.
- Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313.
- Talavera G, Dincă V and Vila R (2013) Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. *Methods in Ecology and Evolution* **4**, 1101–1110.
- Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Kusber W-H, Li D-Z, Marhold K, et al. (eds) (2018) *International Code of Nomenclature for Algae, Fungi, and Plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017*. Glashütten: Koeltz Botanical Books.
- Vainio EA (1922) Lichenographia fennica II. Baeomyceae et Lecideales. *Acta Societatis pro Fauna et Flora Fennica* **53**, 1–340.
- White TJ, Bruns T, Lee S and Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds), *PCR Protocols: a Guide to Methods and Applications*. New York: Academic Press, pp. 315–322.
- Yule GU (1925) A Mathematical Theory of Evolution, Based on the Conclusions of Dr. J. C. Willis, F.R.S. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **213**, 21–87.
- Zahlbruckner A (1905) Ascolichenes. Lieferung 3. In Engler A and Prantl K (eds), *Die Natürlichen Pflanzenfamilien nebst ihren Gattungen und wichtigeren Arten insbesondere den Nutzpflanzen 1* (\*). Leipzig: Engelmann, pp. 97–144.
- Zhang J, Kapli P, Pavlidis P and Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* **29**, 2869–2876.
- Zoller S, Scheidegger C and Sperisen C (1999) PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming ascomycetes. *Lichenologist* **31**, 511–516.